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HLA-E contributes to an immune-inhibitory phenotype of glioblastoma stem-like cells

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Abstract

Cancer stem cells are an attractive target for immunotherapeutic approaches to glioblastoma. However, an immune inhibitory phenotype of cells currently classified as “glioma-initiating cells” (GIC) might counteract recognition by immune effector cells. Here, we investigate the contribution of the non-classical MHC molecule HLA-E to the immunosuppressive phenotype of GIC. HLA-E is expressed in GIC lines and its expression is reduced upon differentiation of GIC in serum-containing culture conditions. Constitutive HLA-E inhibits natural killer (NK) cell-mediated lysis of GIC since small-interfering RNA-mediated HLA-E gene silencing enhances the immunogenicity of GIC. Increased GIC lysis was observed both in the CD133+ and in the CD133- compartment. Furthermore, the use of interferon-γ as a possible agent to boost an immune response against glioblastoma cells might be limited by the concurrent upregulation of HLA-E.

Key words: stem cell; glioblastoma; immunotherapy; HLA-E; NK cell

1. Introduction

Considering the poor prognosis of patients with glioblastoma, there is a need for new therapies beyond surgery, radiotherapy and chemotherapy. Immunotherapy has been considered a promising alternative to treat this devastating disease. However, to date, the clinical results of immunotherapeutic strategies in glioblastoma have remained disappointing. This lack of efficacy might be related to immune inhibitory properties of glioblastoma cells facilitating immune evasion (Heimberger and Sampson, 2011). Glioma cells should per se be prone to attack by immune effector cells since they express ligands for activating immune receptors on NK cells or cytotoxic T cells (Dietrich et al., 2011; Eisele et al., 2006; Friese et al., 2003; Friese et al., 2004; Wischhusen et al., 2005). However, an immunosuppressive micromilieu hampers an efficient immune response. The cytokine transforming growth factor (TGF)-ß contributes largely to this immunosuppression by multiple mechanisms, including upregulation of the inhibitory receptor CD94/NKG2A on immune effector cells (Bertone et al., 1999). This heterodimeric receptor consisting of CD94 and either NKG2A or its splice variant NKG2C is expressed on the cell surface of NK cells as well as CD8+ αβ and γδ T cells (Pietra et al., 2009). The binding of CD94/NKG2A to its ligand, the atypical human leukocyte antigen (HLA-)-E leads to phosphorylation of immunoreceptor tyrosine based inhibitory motifs (ITIMS) in the intracytoplasmatic domain of NKG2A and thus to inhibition of the lytic activity of NK cells. Conversely, the binding of CD94/NKG2C to HLA-E provides an activating signal, however NKG2C binds HLA-E with low affinity and is therefore thought to play a subordinate role (Wada et al., 2004). Under physiological conditions, HLA-E is mainly involved in maternal immune tolerance. HLA-E is abberantly expressed in several tumors like

lymphoma (Marin et al., 2003), melanoma (Derre et al., 2006), colon carcinoma (Bianchini et al., 2006), ovarian cancer (Malmberg et al., 2002) and may contribute to immunosuppression in glioblastoma (Mittelbronn et al., 2007; Wischhusen et al., 2005).

A crucial issue for effective cancer immunotherapy is the definition of the target. In this regard of special interest are cells within glioblastomas with stem cell-like properties. GIC are defined by their stem cell properties of self-renewal, multipotency and tumorigenicity at low cell numbers in immunodeficient mice, forming tumors resembling the initial human tumors (Singh et al., 2004; Tabatabai and Weller, 2011). In a hierarchical tumor model, GIC are supposed to have important functions in the initiation and maintenance of glioblastomas and therefore warrant being evaluated as a possible target for immunotherapy. GIC can be isolated from gliomas *ex vivo* and propagated under serum-free stem cell culture conditions including fibroblast and epidermal growth factor (Gunther et al., 2008; Hemmati et al., 2003). Other approaches to isolate GIC use functional assays or cell surface markers like CD133 (Tabatabai and Weller, 2011). CD133, or prominin-1, a cholesterol binding molecule of unknown biological function, was initially introduced as a putative cell surface marker for GIC (Singh et al., 2004) and is still widely used. However, the role of CD133 in identifying GIC remains controverseial and is challenged by the characterisation of CD133 negative glioblastoma cells with stem cell properties (Beier and Beier, 2011) or the upregulation of CD133 under hypoxic culture conditions or cellular stress (Bar et al., 2010; Griguer et al., 2008).

Here, we investigate the possible contribution of HLA-E to the immune inhibitory phenotype of GIC in a set of previously characterized GIC lines, cultured under stem cell conditions and harbouring the stemness properties of self-renewal, multipotency and tumorigenicity *in vivo* (Gunther et al., 2008). We find cell surface expression of

HLA-E in 4 out of 5 cell lines. HLA-E inhibits NK cell function as demonstrated by increased NK cell mediated cytotoxicity following RNA-mediated HLA-E gene silencing. HLA-E expression is increased by interferon (IFN)-γ, potentially limiting the usefulness of this immune stimulating cytokine in the context of immunotherapy for glioblastoma targeting GIC.

2. Materials and Methods

Materials and Cell Lines

The GIC lines GS-2, GS-4, GS-5, GS-7 and 9 have been characterized (Gunther et al., 2008): in summary, the cell lines expressed stemness markers Sox2 and nestin in undifferentiated GIC and either neuronal (MAP2 and neurofilament), oligodendroglial (galactocerebroside) or glial markers (glial fibrillary acidic protein) in their differentiated counterparts. Moreover, the GIC lines were characterized by self-renewal and their tumorigenicity was confirmed *in vivo*. Gene expression analysis revealed 2 different subtypes of the GIC lines. The GIC cluster 1 (GS-5 and 9) was characterized by the expression of neurodevelopmental genes and a full stem-like phenotype and cluster 2 (GS-2, 4 and 9) by an expression signature enriched for extracellular matrix-related genes with a restricted stem-like phenotype.

All GIC lines were cultured in 75cm^2 culture flasks and maintained in Neurobasal Medium with B-27 supplement (20 µl/ml) and Glutamax (10 µl/ml) from Invitrogen (Basel, Switzerland) and fibroblast growth factor (FGF)-2, epidermal growth factor (EGF) (20 ng/ml each; Peprotech, Rocky Hill, Pennsylvania) and heparin (32 IE/ml; Ratiopharm, Ulm, Germany). GS-2 and GS-5 grow as sphere cultures, GS-7 and GS-9 as semi-adherent and GS- 4 adherent under stem cell medium conditions (Figure 1). GIC lines were differentiated by culture in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Basel, Switzerland), containing 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), penicillin (100 IU/ml)/streptomycin (100 mg/ml) (Gibco) and 10% fetal calf serum (FCS; PAA, Vienna, Austria). The colon carcinoma cell line HT29 served as a positive control for the expression of CD133 (Ieta et al., 2008) and was cultured in DMEM (Invitrogen) containing 10% FCS (PAA), 2 mM L-glutamine (Gibco Life Technologies) and penicillin (100 IU/ml)/streptomycin (100 mg/ml) (Gibco). The NK cell line NKL (Robertson et al., 1996) was cultured in RPMI 1640

medium (PAA, Wien, Austria) containing 15% FCS, 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), penicillin (100 IU/ml)/streptomycin (100 mg/ml) (Gibco), 1 mM sodium pyruvate and 50 U/ml Interleukin 2 (Peprotech).

Monoclonal Antibodies and Flow Cytometry

GIC growing as spheres were mechanically dissociated by pipetting and dissolved in Accutase (PAA) to get a single cell suspension. (Semi-)adherent cells were detached using Accutase. Cell surface expression was assessed with the following monoclonal antibodies (mAbs): anti-HLA-E (clone 3D12; purified and conjugated (allophycocyanine; APC); mouse IgG1, eBioscience, Vienna, Austria), anti-MHC-I and anti-HLA-G (clone W6/32 and 87G respectively; mouse IgG2a, Biologend, Uithoorn, Netherlands), anti-CD133 (clone AC133; PE-conjugated; mouse IgG1, Miltenyi Biotec, Bergisch Gladbach, Germany). Unconjugated IgG1 (clone MOPC 21; Sigma-Aldrich, Buchs, Switzerland) and IgG2a (clone MOPC-173; Biologend) or conjugated IgG1 (clone MOPC 21; PE-conjugated, Sigma-Aldrich) and IgG1 (clone P3.6.2.8.1; APC-conjugated, eBioscience) isotype-matched mAbs were used as controls. The PE-conjugated goat anti-mouse IgG (dilution 1:20) (Dako, Freiburg, Germany) was used as secondary antibody where appropriate. The cells were preincubated in PBS with 2% FCS and stained with specific mAb or matched mouse Ig isotype for 30 min on ice, followed by incubation with the PE-conjugated secondary antibody for 30 min where appropriate. Then the cells were washed with PBS containing 2% FCS. For the detection of HLA-E on CD133-expressing cells, the GIC were double-stained with PE-conjugated anti-CD133 and APC-conjugated anti-HLA-E antibody or matching isotype controls respectively. Flow cytometry was performed with a CyAn ADP Analyzer (Beckman Coulter, Nyon, Switzerland). Specific

fluorescence indices (SFI) were calculated by dividing median fluorescence obtained with the specific antibody by median fluorescence obtained with the control antibody. Following staining with PE-conjugated anti-CD133 mAb, GS-2 cells were separated by fluorescence activated cell sorting (FACS) using the FACSAria III and FACSDiva software (both Becton Dickinson, San Diego, CA) for the presence or absence of CD133 on the cell surface. The purity of the sorted cells was ascertained by subsequent analysis by flow cytometry using a CyAn ADP Analyzer (Beckman Coulter).

Real-Time PCR

RNA was prepared from the cell lines using NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). Subsequently, cDNA was generated using SuperScript II Reverse Transcriptase (Invitrogen AG, Basel, Switzerland). RNA and DNA concentrations were determined using NanoDrop (Thermo Scientific, Waltham, Massachusetts).
cDNA amplification was monitored using SYBRGreen chemistry on the ABI PRISM7000 Sequence Detection System (Applied Bio-Systems, Weiterstadt, Germany) using the following specific primers (forward/reverse): GAPDH: 5'-CTCTCTGCTCCTCCTGTTCGAC-3' (450-469), 5'-TGAGCGATGTGGCTCGGCT-3' (636-619); HLA-E: 5'-GGGACACCGCACAGATTTT-3' (266-284), 5'CTCA-GAGGCATCATTTGACTTTT (519-497) (Wischhusen et al., 2005). Data analysis was done using the DDCT method for relative quantification. Briefly, threshold cycles (CT) for GAPDH RNA (reference) and HLA-E (sample) were determined in duplicate. The expression levels were determined following the formula: $rI=2^{-[C_T HLA\text{-}E - C_T GAPDH]}$.

RNA interference

To silence HLA-E expression, the glioma cells were transiently transfected with siRNA targeting specifically HLA-E (Wischhusen et al., 2005): (535-553): 5'-GCCUACCUGGAAGACACAU(dTdT)-3' and 5'AUGUGUCUUCCAGGUAGGC(dTdT)-3'. ON-TARGETplus Non-Targeting Pool siRNA (Dharmacon, Lafayette, Colorado) was used as negative control. Glioma cells were seeded in 6 cm plates over night and subsequently transfected with siRNA using Metafectene Pro (Biontex, Martinsried, Germany). Gene silencing was verified by flow cytometry at various time points after transfection.

Cytotoxicity assay

We used a flow cytometry-based cytotoxicity assay (Lee-MacAry et al., 2001) which employs the membrane dye PKH-26 and the viability dye ToPro3. This assay is widely used as a system to determine NK or T cell-mediated cytotoxicity. The target cells were stained with PKH-26 membrane dye. This enables the discrimination of the target cells from the effector cells in the subsequent lysis assay. Briefly, the target cells were incubated with PKH-26 dissolved in Diluent C (1:250; all from Sigma–Aldrich) for 3 min. Staining was stopped by washing with RPMI 1640 medium containing 10% FCS. Then the effector and target cells were coincubated at varying effector to target (E:T) ratios for 3 h. Immediately prior to flow cytometry, the viability dye ToPro3 (Invitrogen) was added. The percentage of target cell lysis was determined by flow cytometry using the CyAn ADP Analyzer (Beckman Coulter). Specific lysis was assessed by subtraction of background lysis.

Statistical analysis

The experiments were performed 2 to 3 times as indicated. Statistical significance was calculated by student`s two-tailed t-test where indicated (* $p<0.05$; ** $p<0.01$).

3. Results

GIC express HLA-E

The expression of HLA-E in the GIC lines was assessed on mRNA and protein level using real-time PCR and flow cytometry, both under stem cell conditions and after differentiation. Compared to stem cell conditions, HLA-E mRNA levels were similar or increased in differentiated cultures (Figure 2A). However, on the protein level, we found significant higher levels of cell surface HLA-E in the GIC lines GS-4, GS-5 and GS-7 when grown under stem cell conditions. The SFI values for HLA-E expression in the cell lines GS-2 and GS-9 were similar under stem cell and differentiating culture conditions (Figure2B) with only marginal expression of HLA-E on the surface of GS-9 cells. (Figure 2B and D). Figure 2D shows representative histograms documenting the expression of HLA-E on GIC under stem cell and differentiation conditions. The expression pattern of HLA-E was paralleled by a trend to elevated expression of MHC-I on the cell surface in GS-4, GS-5 and GS-7 under stem cell conditions (Figure 2C).

HLA-E gene silencing in GIC enhances susceptibility towards NK-cell mediated lysis

To define the role of HLA-E in immune recognition of GIC by NK cells, we used siRNA to transiently silence HLA-E expression in GS-2, GS-5 and GS-7 growing under stem cell medium conditions. The knock-down of HLA-E was confirmed by flow cytometry (Figure 3A). The expression level of MHC-I remained unaltered following HLA-E gene silencing as confirmed by flow cytometry (data not shown). Following the knock-down of HLA-E expression, the GS cells were then used as targets in a cytotoxicity assay using NKL as effector cells. The knock-down of HLA-E led to an

enhanced susceptibility of GIC to NK cell-mediated lysis (Figure 3B). This was also true for GS-7 cells growing under differentiation medium conditions (data not shown).

HLA-E cell surface expression on GIC is up-regulated by IFN-γ

Stimulation of GIC with IFN-γ (500 IU/ml) for 48 h led to enhanced expression of HLA-E mRNA as assessed by real-time PCR in GS-5, GS-7 and GS-9 and to a lesser extent in GS-2 and GS-4 (Figure 4A). Conversely, the cell surface expression of HLA-E protein was increased in all GS lines (Figure 4B), paralleled by an increase of MHC-I expression (data not shown). The upregulation of HLA-E on the cell surface was similar on GIC growing under stem cell or differentiation medium conditions except for GS-5 with a prominent increase of HLA-E following treatment under stem cell compared to differentiation conditions (Figure 4B).

To assess a possible immune-inhibitory effect and to determine the net immune-regulatory effect following stimulation of GIC with IFN-γ, the cell lines GS-5 and GS-7 were used as targets in a cytotoxicity assay using NKL as effector cells. While IFN-γ had no effect on the susceptibility to NKL mediated lysis in GS-7 (Figure 4D), the lytic activity of NKL towards GS-5 treated with IFN-γ was reduced compared to untreated controls (Figure 4C).

Silencing of HLA-E expression in CD133+ cells enhances susceptibility towards NK-cell mediated lysis

Despite limitations, CD133 is still used as a putative cell surface marker for GIC. Therefore, we assessed cell surface expression of HLA-E on CD133+ and CD133- cells and the impact of HLA-E gene silencing on NK cell mediated lysis by absence or presence of CD133 in the GIC line GS-2 (Figure 5). As reported (Gunther et al., 2008), approximately 9% of the GS-2 cells are CD133+ as assessed by flow

cytometry. The expression of HLA-E was similar on CD133+ and CD133- GS-2 cells as assessed by flow cytometry following double staining with PE-conjugated anti-CD133 and APC-conjugated anti-HLA-E mABs (Figure 5A). The GS-2 cells were sorted by FACS into a CD133+ and a CD133- fraction using the FACSAria III device. Purity of the respective fractions was ascertained with about 95% by flow cytometry. Following sorting, we used siRNA to transiently silence HLA-E expression in CD133+ and CD133- GS-2 cells as described above. The knock-down of HLA-E was confirmed by flow cytometry (data not shown). Following the silencing of HLA-E expression, the cells were used as targets in a cytotoxicity assay using NKL as effector cells. The knock-down of HLA-E led to an enhanced susceptibility of GS-2 GIC to NK cell-mediated lysis irrespective of the expression of CD133 (Figure 5B).

To investigate a possibly differential regulation of HLA-E expression by IFN-γ, a fraction of the sorted cells was treated with IFN-γ (500 IU/ml for 48 h). Following stimulation with IFN-γ the cell surface expression of HLA-E was increased in CD133+ (SFI 9.4 vs. SFI 2.4 in untreated controls) and CD133- (SFI 9.7 vs. 2.3 in untreated controls) cells as assessed by flow cytometry.

4. Discussion

Immunotherapeutic approaches to glioblastoma continue to attract significant attention, based on their localized growth in the central nervous system and the associated cellular immunosuppression. Recent reports have defined an immune-inhibitory phenotype specifically of GIC (Di Tomaso et al., 2010; Wei et al., 2010a; Wei et al., 2010b). Here, we define a possible role of the atypical HLA molecule, HLA-E, for the immunosuppressive phenotype of GIC. HLA-E is expressed on the cell surface of all GS lines investigated here, except for GS-9 (Figure 2B). Compared to differentiating culture conditions, the expression of HLA-E is enhanced under stem cell conditions in GS-4, GS-5 and GS-7 (Figure 2B). The cell surface expression of HLA-E under stem cell compared to differentiating conditions is not reflected at the level of mRNA expression (Figure 2A and B). Therefore, other mechanisms regulating HLA-E expression on GIC seem to be involved. Similar to classical MHC class-I molecules, the expression of HLA-E depends on efficient peptide loading leading to the assembly of a trimeric complex including beta2-microglobulin and subsequent translocation to the cell membrane (Ulbrecht et al., 1999). This process can be compromised in tumor cells, leading to differences between HLA-E mRNA and cell surface expression levels of HLA-E (Palmisano et al., 2005). Moreover, metalloproteinase-dependent shedding of HLA-E occurs in tumor cells (Derre et al., 2006). Therefore, the expression of HLA-E on the cell surface in GIC might be under the control of complex post-translational regulation.

The immunosuppressive mechanisms of GIC include induction of regulatory T cells, inhibition of proliferation and apoptosis of T cells *in vitro* with B7-H1 and soluble galectin-3. These immunosuppressive functions of GIC seem to depend on the constitutive activation of the STAT-3 pathway, a potent anti-inflammatory regulatory pathway (Wei et al., 2010a). Moreover, a defective antigen processing machinery in

GIC underscores their ability to evade a T cell-mediated immune response (Di Tomaso et al., 2010). However, GIC can not entirely hide from immune effector cells since they can be attacked by NK cells via the activating receptors NKp46, DNAM-I or to a lesser extent NKG2D *in vitro* (Castriconi et al., 2009; Di Tomaso et al., 2010), a process which might be counteracted by HLA-E.

Here, we provide evidence that HLA-E hampers indeed NK cell-mediated immune recognition of GIC as demonstrated by siRNA-mediated gene silencing (Figure 3B, 5B), thus contributing to the immunosuppressive phenotype of GIC. These results are consistent with the role of HLA-E in counteracting NK cell-mediated cytotoxicity towards human glioma cell lines (Wischhusen et al., 2005).

The role of HLA-E in the regulation of anti-tumor immune responses is mainly investigated in the context of innate immunity given the predominant expression of the receptors NKG2A and NKG2C on NK cells. However, these receptors are also expressed on a subset of cytotoxic T lymphocytes (Arlettaz et al., 2004). Little is known about interactions of HLA-E with T lymphocytes in glioblastoma. The correlation of HLA-E expression levels with the extent of infiltration by CD8+ T cells in human glioblastoma might be explained with an IFN-γ induced upregulation of HLA-E (Mittelbronn et al., 2007). However, restrained function of tumor-infiltrating CD8+ T lymphocytes as a consequence of tumor-associated expression of HLA-E has been demonstrated in ovarian cancer (Gooden et al., 2011). Moreover, HLA-E can bind and present viral or bacterial peptides and interact directly with the T cell receptor of HLA-E restricted CD8+ T cell subsets, thus regulating adaptive immunity against infectious pathogens (Pietra et al., 2003; Schulte et al., 2009). However, the role of this mechanism in tumor immunity is yet not clear. Peptides derived from isoforms of peroxiredoxin 5, occurring in a melanoma cell line, were recognized by CD8+ HLA-E-restricted T lymphocytes. In contrast, this HLA-E-peptide complex failed to activate

the inhibitory receptor NKG2A (Sensi et al., 2009). This suggests even the possibility of an anti-tumor immune mechanism exerted by HLA-E by presenting tumor-specific peptides to cytotoxic T lymphocytes, a mechanism which has not been proven yet. A role for HLA-E in regulating adaptive immunity in glioblastoma warrants further investigation.

The overall role of HLA-E in glioblastoma biology still needs to be defined. The expression of HLA-E was elevated in pilocytic astrocytomas (WHO grade I) and glioblastoma (WHO grade IV) compared to gliomas of WHO grades II and III, but the expression of HLA-E was not associated with patient survival (Mittelbronn et al., 2007). In contrast, another recent report describes a counter-intuitive positive prognostic correlation of HLA-E expression in human glioblastomas *ex vivo* with survival (Kren et al., 2011), a finding which might be related to subsequent therapies following tumor resection. However, in other tumor entities like breast (de Kruijf et al., 2010) or colorectal cancer (Levy et al., 2008), enhanced expression levels of HLA-E were correlated with reduced survival. To elucidate the role of HLA-E in glioblastoma it might be promising to investigate HLA-E expression on cell subtypes within gliomas, like tumor infiltrating microglia (Kren et al., 2010). In this regard, the role of HLA-E expression on GIC *in vivo* for the survival of the patients warrants further investigation.

The expression of MHC class-I molecules and therefore the immune recognition by CD8+ T cells are diminuished in gliomas as one mechanism of immune escape (Facoetti et al., 2005). In turn, reduced MHC class-I expression would lead to enhanced immune recognition by NK cells. IFN-γ is often used to enhance MHC class-I expression. In turn, in several tumor entities like leukemia, ovarian cancer or melanoma, HLA-E is upregulated on the cell surface upon treatment with IFN-γ

(Derre et al., 2006; Malmberg et al., 2002; Nguyen et al., 2009) which might protect these tumor cells from immune effector cell-mediated lysis.

Here, we demonstrate the upregulation of HLA-E on a transcriptional level and on the cell surface following treatment with IFN-γ in glioblastoma cells (Figure 4A and B). However, IFN-γ exerts multiple effects in glioblastoma cells including the up-regulation of MHC class-I and –II and co-stimulatory molecules, but also glioma the reduction of proliferation and invasion (Haque et al., 2007; Kane and Yang, 2010). In contrast, the upregulation of atypical MHC class-I molecules HLA-E and HLA-G (Wischhusen et al., 2007) and other immunosuppressive molecules like B7-H1 (Han et al., 2009) upon treatment with IFN-γ may serve as one further immune escape mechanism of glioblastoma cells. Having multiple, in part antidromic actions in glioma cells, the net effect of IFN-γ on the immunophenotype of GIC is crucial and might vary between patients. This is exemplified in Figure 4 with reduced (Figure 4C) or unchanged (Figure 4D) immunogenicity in GS-5 and GS-7 respectively upon treatment with IFN-γ. Therefore, the use of IFN-γ to boost an immune response against GIC might be limited.

In this work, we used a panel of well characterized GIC lines, kept under stem cell culture conditions. Although controversially discussed (Beier and Beier, 2011; Tabatabai and Weller, 2011), CD133 is still used as a putative cell surface marker for GIC. Therefore, we assessed the role of HLA-E specifically on CD133+ cells exemplarily in the GIC line GS-2. The expression level of HLA-E was similar in CD133+ and CD133- GS-2 cells (Figure 5A). The siRNA-mediated silencing of HLA-E expression enhanced NK cell-mediated immune recognition of CD133+ and CD133- GS-2 cells in a similar manner (Figure 5B). Together with the demonstration of enhanced expression of HLA-E on the surface of CD133+ cells upon treatment with IFN-γ, we thus reproduce the findings for the role of HLA-E in GIC lines in cells

sorted for the presence of CD133. Overall, HLA-E contributes to the immunosuppressive properties of GIC, but has to be considered in the context of a complex network of immune regulatory influences exerted by these cells.

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6. Figure legends

Fig. 1: **Culturing of GIC.** The GIC lines (as examples GS-4, GS-5 and GS-9) were cultured in stem cell-permissive neurobasal medium containing FGF-2, EGF, B27 supplement and heparin. The scale bars are shown in the lower right corner.

Fig. 2: **HLA-E is differentially expressed under stem cell culture and serum-containing conditions.**

HLA-E mRNA expression was analyzed by real-time PCR under stem cell culture (A, open bars) or differentiated conditions (B, GS-Xd, black bars). Data are expressed relative to GAPDH expression. One representative experiment is shown (n=2). (B-D) GIC and differentiated cultures were stained with mAbs for HLA-E (B and D; clone 3D12), MHC-I (C; clone W6/32) or isotype-matched Ig, followed by staining with a PE-labelled anti-mouse secondary antibody. In B and C data represent mean SFI values ± SEM from 3 independent experiments (* $p<0.05$; student`s two tailed t-test). In (D) SFI values are indicated in the upper right corner and are representative of three independent experiments (curve in light grey: isotype control, dark grey specific mAb).

Fig. 3: **HLA-E gene silencing enhances susceptibility of GIC to NK cell-mediated lysis.**

(A) The GIC lines GS-2, GS-5 and GS-7 were transfected with control (siCtrl) or siRNA targeting HLA-E (siHLA-E). The cells were harvested 36 h later and knock-

down efficiency was confirmed by flow cytometry (curve in light grey: isotype control, dark grey: specific HLA-E mAb, clone 3D12).

(B) The cells transiently transfected with siCtrl or siHLA-E were used as targets in a cytotoxicity assay using NKL cells as effectors. Target cells were stained with the membrane dye PKH-26 and then co-cultured with NKL cells at various effector to target (E:T) ratios for 3 h in duplicates. The cells were stained with the viability marker ToPro3 to identify the lysed cells by flow cytometry. Specific lysis was calculated by substraction of background lysis from the fraction of dead cells. One representative experiment is shown (n=2 to 3).

Fig. 4: **The expression of HLA-E on GIC cell surface is enhanced by IFN-γ.**

GIC and corresponding differentiated cell cultures were incubated with IFN-γ (500 IU/ml) for 48 h. (A) HLA-E mRNA expression was analyzed by real-time PCR. Data are expressed relative to GAPDH expression. One representative experiment is shown (n=2).

(A) GIC and corresponding differentiated cell cultures were stained with mAbs for HLA-E (B; clone 3D12), MHC-I (C; clone W6/32) or isotype-matched Ig, followed by staining with a PE-labelled anti-mouse secondary antibody. Data represent SFI mean values ± SEM from 3 independent experiments (* $p<0.05$; student`s two tailed t-test).

(C), (D) The GIC cell lines GS-5 and GS-7 were used as target cells in a cytotoxicity assay with NKL cells as effectors after stimulation with IFN-γ as described above. One representative experiment is shown (n=3)

Fig. 5: **NK cell-mediated lysis of GS-2 cells is independent of CD133 expression.**

(A) HLA-E cell surface expression on GS-2 GIC was assessed by flow cytometry following staining with anti-HLA-E (APC) and anti-CD133 (PE) or suitable isotype

controls. SFI values for HLA-E expression on cells gated for the presence or absence of CD133 are indicated in the upper right corner of the histograms which are representative of 2 independent experiments (curve in light grey: isotype control, dark grey: specific mAb; FSC: forward scatter). (B) Following FACS sorting of GS-2 cells for the presence or absence of CD133 cell surface expression, the cells were transfected with control (siCtrl) or siRNA targeting HLA-E (siHLA-E) and used as targets in a cytotoxicity assay using NKL cells as effectors as described above. One representative experiment is shown (n=2).